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Cytoskeletal analysis of primitive mouse neuroectodermal cells [1]

by

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With 4 figures

Abstract

The cytoskeleton of the 9.5 BC cell line derived from embryonic mouse brain and isolated mesencephalon-rhombencephalon samples of mouse embryos at 10.5 days of gestation has been examined by immunohistochemistry, electrophoretic separation and immunoblotting techniques. The cell line is composed of a high proportion of cells positive for glial fibrillary acidic (GFA) protein and, to a lesser degree, myelin basic (MB) protein. Therefore it is considered to be a gliogenic cell line. The cytoskeleton of the proliferating cells is composed of actin, vimentin and several cytokeratin polypeptides (54 and 57 kD acidic and 61 kD basic polypeptides). The cytoskeleton of the embryonic brain is composed of several cytokeratin polypeptides (40 and 46 kD acidic and 54, 57, 61, 63 and 68 kD basic components), the predominant component being the 46 kD acidic cytokeratin. Vimentin is already detectable between the 10th and 11th day of embryonic life. As the glial cells of the central nervous system arise from a cytokeratin positive cell population and switch their cytoskeletal composition to a cytokeratin negative phenotype, any comprehensive explanation of this histogenetic process should take into account the repression of cytokeratin polypeptide synthesis during ontogeny. As the 9.5 BC cell line is a cytokeratin positive and embryonic brain derived line with the developmental potency to produce glia cells, we propose it as an *in vitro* model suitable for the study of this developmental step.

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INTRODUCTION

The whole variety of nerve, macroglial and ependymal cells constituting the vertebrate central nervous system are the descendants of an embryonic structure, called the neural tube. This structure and the ventricular zone of the developing central nervous system appear as a pseudostratified epithelium composed of radially oriented columnar cells. All ventricular cells divide, giving rise to the ventricular zone with a homogeneous cell population (FUJITA 1965; ANGEVINE 1970; SIDMAN 1970). In spite of the morphological and cytokinetic characterization, much less is known about the biochemical constitution of a ventricular zone and its cells. The aim of the present study was to gain information about the biochemical properties of the early neuroectodermal tissue of the mouse. For this analysis we have chosen mesencephalic-rhombencephalic sections of the 10.5 days old mouse embryo as representative sample of the ventricular zone. The reason for our choice is based on several facts. Neurulation in the mouse embryo processes during the 8th to 9th day of embryonic life (THEILER 1972). Following the next day, the mesencephalon constitutes a considerable portion of the whole embryo, so that enough material can be isolated from a few embryos for analysis. At this stage of development, this particular region of the brain is composed of a single ventricular zone. A well detectable marginal zone together with the incipient intermediate zone (ANGEVINE *et al.* 1970) develops later during the 11th day. It is also an important feature that the epidermis, most of the surrounding mesenchyme and the neighbouring brain sections can easily be separated mechanically. The same advantage holds true for the 9.5 days old embryo although it is about half the size (THEILER 1972) of the 10.5 days old one. As an alternative approach we isolated a cell line from the 9.5 days old embryonic brain in order to have an easily accessible source of material *in vitro*. This cell line and its possible application as a model for the progenitor neuroectodermal cell of the mouse central nervous system will be described here.

MATERIALS AND METHODS

Isolation and cultivation of cells from embryonic brain

Female mice of the C57BL/10 strain were mated overnight to males of the same strain. The next morning, females with vaginal plugs were selected. The day of the vaginal plug was counted as day 0 of gestation. To initiate the cell line embryos were collected from a C57BL10 female at 9.5 days of pregnancy. The embryos were at stage 15 (THEILER 1972). The head region was separated, the epidermis and the mesectoderm were mechanically dissected from the continuous tube of the mesencephalon-rhombencephalon "anlage", and finally the telencephalon vesicles were removed. The dissected brain parts were treated with Trypsin-EDTA (0.125% trypsin and 0.125% ethylenediaminetetraacetic acid) for 10 min at 37° C. At the end of incubation the tissue fragments were pipetted extensively to release loosened residual mesenchymal cells. Solid pieces of brain tissue which remained unsuspended after the first enzymatic treatment were collected and digested for another 20 min at 37° C in fresh Trypsin-EDTA solution. After the second digestion the tissue fragments were transferred into drops of tissue culture medium, suspended and incubated under paraffin oil in a 10% CO_2 atmosphere at 37° C. To establish and maintain the *in vitro* cell line as well as the differentiating cultures, Dulbecco's modified Eagle's medium

was used, supplemented with 10% fetal calf serum, 1 mM pyruvate, 100 IU/ml penicillin, 50 IU/ml streptomycin and 5×10^{-5} M 2-mercaptoethanol. Further details concerning the culture procedures will be published elsewhere.

Immunocytochemical analysis

Cytoskeletal polypeptides and myelin basic protein were examined in samples derived from the 9.5 BC cell line before the 50th *in vitro* passage. Several thousand cells were plated onto sterile glass slides and cultivated for various lengths of time (from 4 days to 8 weeks). At the end of the incubation period, the slides with the attached cells were washed with phosphate buffered saline (PBS), air-dried and fixed with methanol for 10 min at −20° C. Before initiating the immunolocalization procedure, the methanol fixed samples were rehydrated in PBS and then incubated in normal horse serum (1: 50 dilution) to saturate the non-specific protein binding sites.

Monoclonal antibodies specifically reactive with glial fibrillary acidic protein (clone G-A-5), neurofilament polypeptides of 68 kD (clone NR4), 160 kD (clone NN18) and 200 kD (clone NE14) were employed (Debus *et al.* 1983). To probe for the presence of cytokeratin polypeptides, a conventional guinea pig anti-keratin antiserum was used at a dilution of 1: 50. Vimentin was detected with a conventional guinea pig antibody (1: 100 dilution) which was generously provided by W. W. Franke (Franke *et al.* 1982). Myelin basic protein expression was detected with a rabbit anti-human MB protein antiserum at a dilution of 1: 40. The antibody was kindly provided by J. M. Matthieu (Bürgisser *et al.* 1982; Bürgisser 1983).

Immunofluorescent detection of these antibodies was carried out with a goat anti-mouse IgG $F(ab')_2$ fragment conjugated with FITC (1: 50 dilution) or a goat anti-guinea pig IgG FITC conjugate (1: 20 dilution) or a goat anti-rabbit IgG FITC conjugate (1: 30 dilution). Immunolocalization with peroxidase was done according to the biotin avidin bridging method (Hsu *et al.* 1981). To detect the mouse derived monoclonal antibodies, the Vectastain ABC kit to mouse IgG was used directly. The detection of guinea pig derived immunoglobulins required the application of goat anti-guinea pig IgG which has been conjugated with biotin-N-hydroxysuccinimide ester (Zákány *et al.* 1984).

Electrophoretic and immunoblotting procedures

Total cellular proteins were collected from cultures of the 9.5 BC cell line after extensive rinsing with serum free medium. Extracts from about 4×10^6 cells were used for electrophoresis. Cytoskeletal proteins from two monolayer cultures were prepared *in situ* as described (Franke *et al.* 1981) and collected for electrophoresis and immunoblotting. The brain of 9.5 and 10.5 days old embryos was isolated as described except that no enzymatic treatment was applied after the mechanical dissection in order to minimize proteolytic degradation of the proteins. Brain extracts derived from 7 to 9 embryos at 10.5 days and from 14 embryos at 9.5 days were loaded onto one gel either for electrophoretic analysis. Total proteins were detected with Coomassie Brilliant Blue G-250 (C.I. 42655).

Immunoblotting analysis was carried out according to standard procedures (Towbin *et al.* 1979) on homogeneous 10% SDS gels (10% T, 2.6% C) with stacking gels (3% T, 2.6% C) on top of the separating gels. Immunochemical detections of polypeptides on the filters were done by applying the same anti-keratin, anti-vimentin, anti-GFA protein, anti-NF 68, 160 and 200 antibodies and the biotin-avidin bridging procedure as described above. The only difference was that the antibodies were diluted in PBS containing 0.05% Tween 20 and 1% bovine serum albumin.

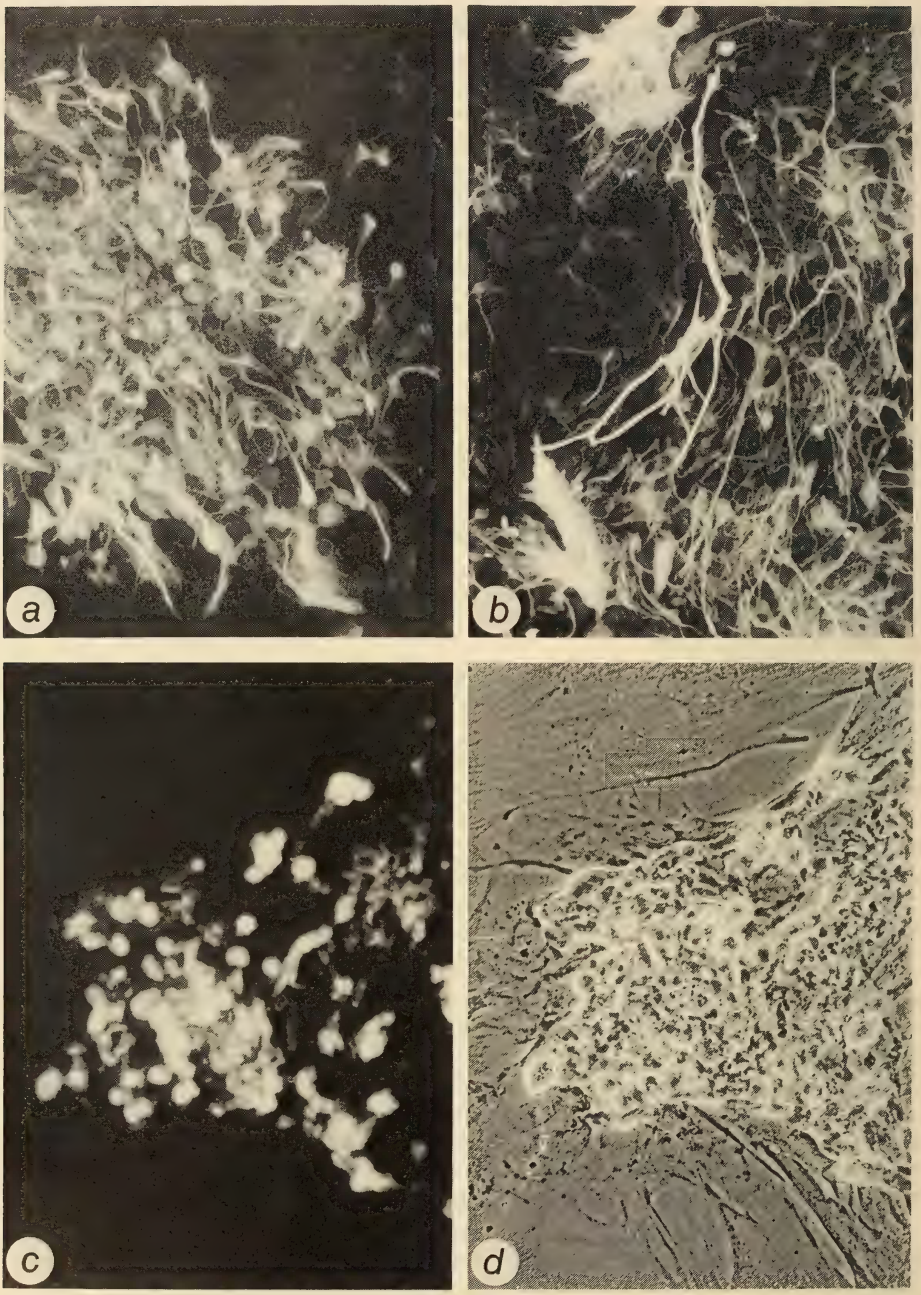

FIGURE 1.

Immunocytochemical analysis of glia cell markers in differentiated cultures of the 9.5 BC line. Indirect immunofluorescent detection of glial fibrillary acidic (GFA) protein in cultures two weeks old (*a*) and three weeks old (*b*), as well as myelin basic (MB) protein in cultures three weeks old (*c*). The phase contrast micrograph (*d*) demonstrates the morphology of MB protein positive cells (the same area as shown in *c*). Magnifications: $a \times 225$, $b\text{-}d \times 360$.

RESULTS

This publication deals with the immunocytochemical detection and biochemical analysis of cytoskeletel components in cells of the 9.5 BC cell line cultured *in vitro* and derived from embryonic mouse brain in comparison with the respective tissue *in situ*. A detailed description concerning the isolation, establishment and further characterization of this apparently diploid cell line with a modal chromosome number of 40 will be published elsewhere.

After one week of culture of these cells, multicellular foci appeared as first signs of morphological differentiation. During the second week, these areas were built up by cells showing a few relatively short processes. Cultures two to three weeks old invariably contained a significant proportion of GFA protein positive cells which first appeared in these dense cellular areas, as detected by the use of a monoclonal anti-GFA protein antibody (Fig. 1*a* and *b*). At the periphery of these areas, the GFA protein negative cells had a contact inhibited epithelial morphology. Cultures three weeks old contained areas of extensive astrocyte differentiation. By this time about half of the cells were GFA protein positive.

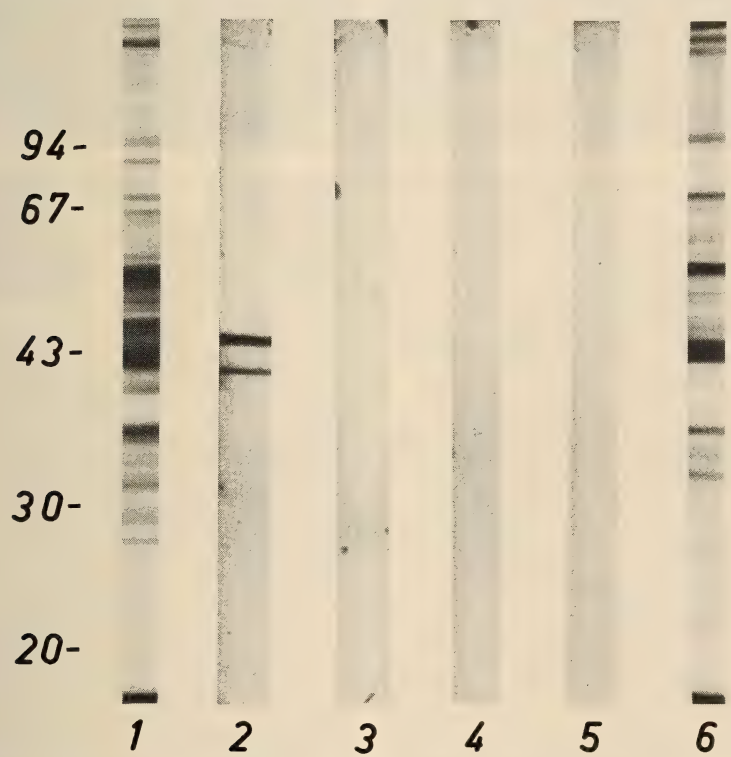

FIGURE 2.

Western blot analysis of total cellular proteins derived from differentiated (lanes 1-3) and nondifferentiated (lanes 4-6) cultures of the 9.5 BC cell line. Lanes 1 and 6 show total cellular proteins stained with Coomassie Brilliant Blue. Lane 2 documents immunodetection with anti-GFA protein monoclonal antibody, which is not the case for lane 4. Lanes 3 and 5 exhibit negative reaction with anti-neurofilament subunit protein monoclonal antibodies.

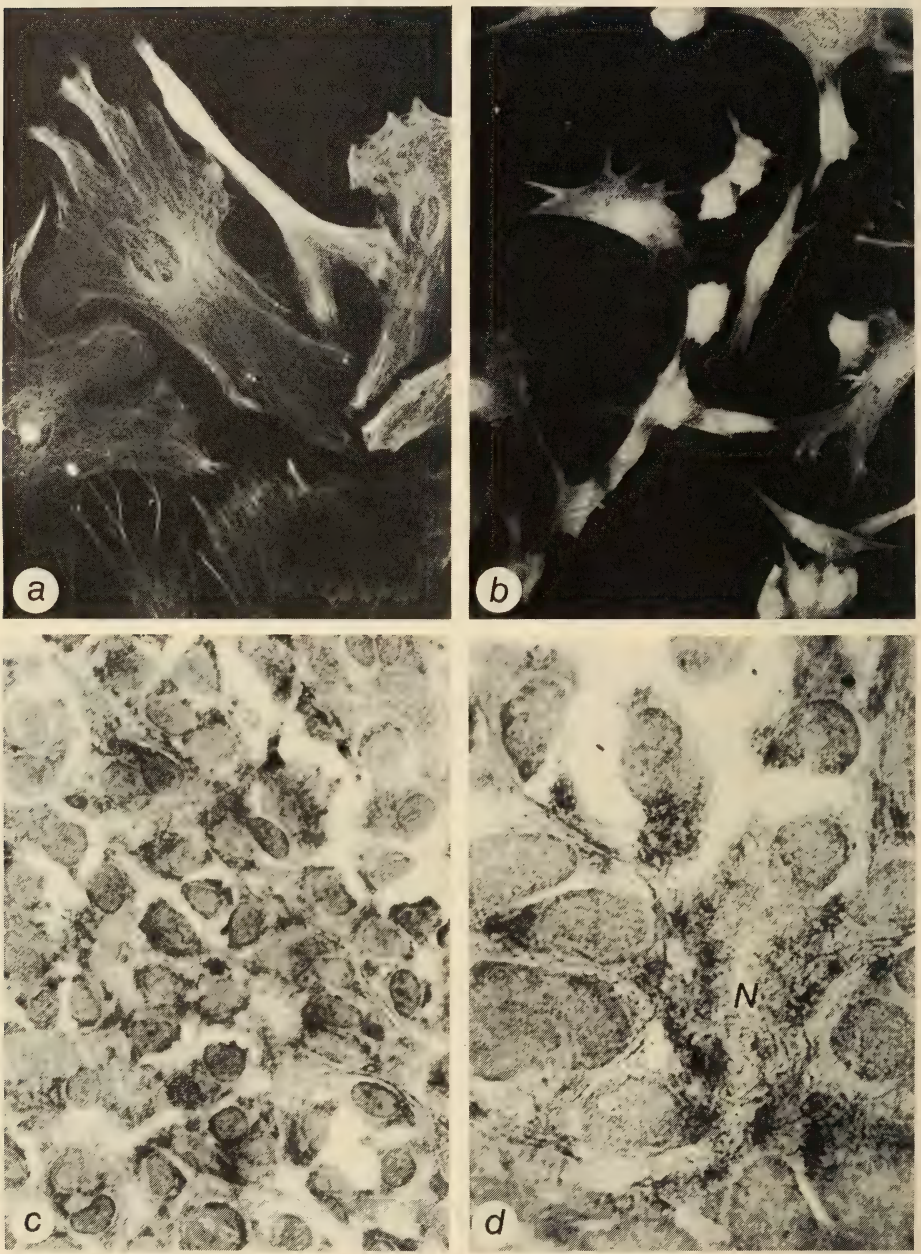

FIGURE 3.

Immunocytochemical detection of vimentin and cytokeratin in the 9.5 BC cell line. Indirect immunofluorescence microscopy of vimentin (*a*) and cytokeratin (*b*) shows the presence of these cytoskeletal proteins in the cultured cells. Immunoperoxidase detection corroborated these findings and revealed cytokeratin positive processes (*c*), which in certain areas (N) form a dense network (*d*). Magnifications: *a* and *b* × 360, *c* × 570, *d* × 1400.

Two main GFA protein positive cell types were seen, one with long cellular processes of variable diameter and the other without long processes but with a heavily stained cytoplasm. At later stages of culture, the GFA protein positive component prevailed whereas the epithelial cells were no longer detectable by the eighth week.

After the third week, some restricted areas arose which showed a different morphology. The epithelial monolayer contained groups of round cells connected by rough thick processes. In cultures in which the cells were plated onto glass slides, these cells appeared usually at the periphery of the outgrowth and reacted to anti-MB protein antibody (Fig. 1*c*). This antigen was detectable on the cell bodies as it is expected from an antigen bound to the cell membrane. No staining of the processes was observed in these cultures (Fig. 1*d*). Immunocytochemical trials to detect any of the three neurofilament subunit polypeptides in the differentiated cultures proved negative at any stage. Since in the differentiated cell population of advanced cultures the majority of the cells was GFA protein positive, we also tried to detect this intermediate filament subunit polypeptide among the electrophoretically separated total cellular proteins, using Western blotting analysis and applying monoclonal antibodies reactive with GFA protein and each of the neurofilament subunit polypeptides (Fig. 2). In accordance with the immunocytochemical observations, specific immune reaction was observed only when proteins from differentiated cultures were probed with anti-GFA protein antibody. The reactive band appeared at 50 kD apparent molecular weight characteristic for the GFA protein intermediate sized filament subunit. No GFA protein specific immune reaction was observed in exponentially growing cultures. Corresponding to the immunocytochemical data, no neurofilament expression could be detected neither in differentiated nor exponentially growing cell populations.

Immunocytochemical detection of vimentin was achieved by indirect immunofluorescence microscopy. Almost every cell was found to be positive, although there were characteristic differences in the intensity and intracellular distribution of fluorescence between different cells. Long cells with relatively sparse cytoplasm exhibited the strongest fluorescence. Many cells exhibited vimentin in fine cytoplasmic cellular extensions that contacted the neighbouring cells (Fig. 3*a*). Immunofluorescence detection of keratin polypeptides gave a similar picture (Fig. 3*b*). Again almost every cell was labeled by the anti-keratin antibody. In general the keratin containing intracellular filaments were thinner than those containing vimentin. The fine cellular extensions which were found to be vimentin positive did also contain keratin immunoreactivity. Immunoperoxidase staining for keratin polypeptides showed cytoplasmic keratin staining in these cells. In higher density cultures a complex reticular background of keratin containing filaments developed (Fig. 3*c*). In certain areas several cells projected these filaments into the same direction forming bundles of whirles (Fig. 3*d*).

Western blotting analysis of separated total proteins from the brain derived cell culture confirmed the presence of an anti-vimentin antibody reactive band at 56 kD relative molecular weight (Fig. 4, lane 1). Immunodetection with anti-keratin antibodies revealed the presence of three bands at 54, 57 and 61 kD relative molecular weight (Fig. 4, lane 2). Similarly, the presence of vimentin (Fig. 4, lane 3) and keratin polypeptides (Fig. 4, lane 4) in the total electrophoretically separated cellular proteins from embryonic brain was detected by immunoblotting. The strongest keratin band was found at 46 kD relative molecular weight. In addition, bands at 54, 57, 61, 63 and 68 kD were detected as well. Further characterization of the cytoskeletal components from the 9.5 BC cultures and embryonic brain were carried out using two-dimensional electrophoresis and will be published elsewhere.

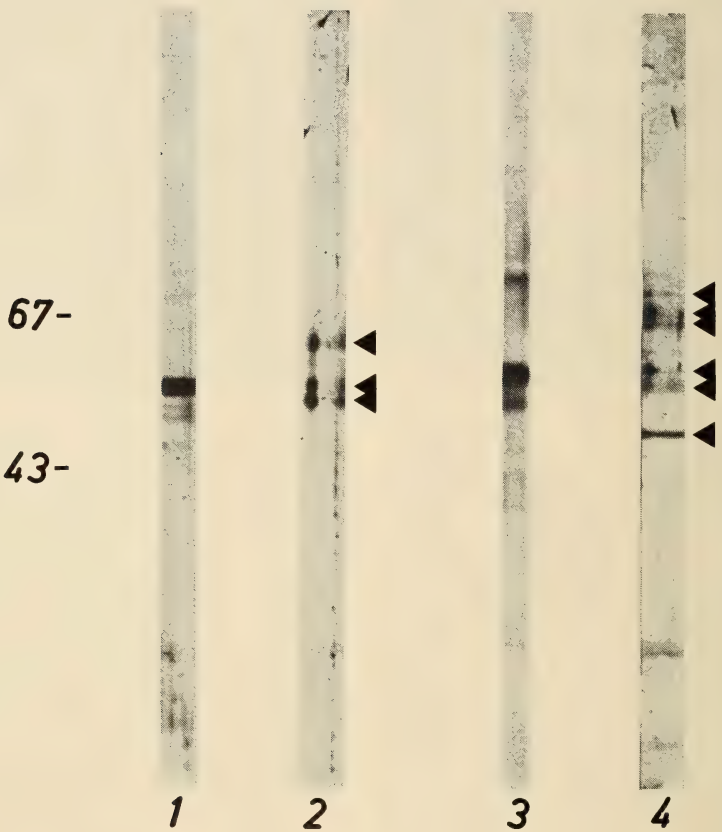

FIGURE 4.

Western blot analysis of total cellular proteins derived from cultures of the 9.5 BC cell line (lanes 1 and 2) and from brain of embryos 10.5 days old (lanes 3 and 4). Lanes 1 and 3 document immunodetection with anti-vimentin antiserum, lanes 2 and 4 exhibit immunodetection with anti-keratin antiserum. Both the cell line and embryonic brain contain vimentin and cytokeratin immunoreactive material at the correct molecular weight range. Vimentin is represented at 56 kD (lanes 1 and 3). Cytokeratins are indicated by arrows at 54, 57 and 61 kD (lane 2), and at 46, 54, 57, 61, 63 and 68 kD (lane 4).

Discussion

This paper deals with the isolation and characterization of a cell line derived from embryonic mouse brain and compares it morphologically and biochemically with the developing brain. In the experiments reported here we have concentrated on its application as a starting material for the biochemical analysis of the ventricular cell. An obvious similarity between the embryonic brain derived cell line and the ventricular cell is that neither of them express glia specific markers but both can give rise to glia cells under certain conditions. In addition, this *in vitro* cell line as well as the ventricular cells of the mouse embryo at 11 days of gestation do express immunohistochemically detectable vimentin (SCHNITZER *et al.* 1981). The presence of vimentin in this *in vitro* cell line has also been discovered by immunoblotting methods, documenting the correct 56 kD relative molecular weight value for the immunoreactive material. In fact, two-dimensional separation of cytoskeletal protein isolates has shown that, apart from residual actin, vimentin is the most abundant cytoskeletal polypeptide component in this cell line.

The analysis of the cytoskeletal extracts has also revealed several minor components, which appear on the two-dimensionally separated polypeptide patterns as some of the already known cytokeratin polypeptides (FRANKE *et al.* 1981; SCHILLER *et al.* 1982). On the basis of the relative localization of the spots to vimentin on the electropherograms, the 54 kD spots seem to correspond to component 13 and 14, and the 57 kD spots to component 12 of mouse derived cytokeratins (SCHILLER *et al.* 1982). The identification of the 61 kD spot is less obvious, but it most probably represents component 5. We have noticed, that the pattern of cytokeratin polypeptides detected in these cultures is variable, since some higher molecular weight basic components sometimes appear as well. However, the precise regulation on this phenomenon needs further studies.

From immunocytochemical data of localizing cytoskeletal polypeptides as well as vimentin in almost every cell of the BC line, we assume that the cells of this BC line coexpress vimentin and cytokeratin polypeptides, although double-labelling experiments have not been performed. On the other hand, the existence of GFA protein negative and vimentin positive glial precursor cells has already been well documented (BIGNAMI *et al.* 1982; DAHL 1981; DAHL *et al.* 1981; RAFF *et al.* 1979; SCHWEIZER & WINTER 1982). Nevertheless, for the time being, we suggest that a precursor cell coexpressing cytokeratin polypeptides and vimentin may also exist. Certainly, further studies, in which cells of the BC line are being double-labelled for vimentin and cytokeratins, are required to verify this proposal. In fact, vimentin could be easily detected among electrophoretically separated proteins of the embryonic brain 10.5 days old. This finding has also been repeatedly observed in the immunocytochemical studies that this cytoskeletal protein is expressed in the ventricular cells of the developing mouse central nervous system, as it has already been described (SCHNITZER *et al.* 1981). We propose that it is expressed in these cells as early as day 10.5 at a stage in development, when the ventricular cell population seems to be histologically homogeneous.

This observation is in contrast to the results obtained in histochemical studies on the early replicating neuroepithelial cells of rat embryos, where vimentin expression was detected only in conjunction with the appearence of the first neurofilament positive young postmitotic neurons (BIGNAMI *et al.* 1982). This contradiction is rather unexpected since in the early neural tube of a phylogenetically more remote species, the chicken, vimentin was easily detectable well before the formation of neurofilament positive cells (TAPSCOTT *et al.* 1981), as it was found to be the case in our studies. We therefore conclude that vimentin is expressed in the neuroectodermal cells during murine embryogenesis, since in

the surrounding mesectodermal and epidermal head tissues no vimentin was seen in our immunohistochemical samples. A possible explanation for the current discrepancy may be that the immunodetection system and the anti-vimentin antibody applied by us is more sensitive to small amounts of vimentin than the detection system used previously in the rat studies. With this interpretation we can maintain the proposition that replicating neuroepithelial cells, the precursors of glia cells and neurons, do express vimentin but not as a major cytoskeletal component. Clearly, further studies are needed to resolve this problem.

The cytokeratin profiles established from the 9.5 BC line and embryonic brain vary from those proposed in previous studies of preimplantation (JACKSON *et al.* 1980; OSHIMA *et al.* 1983) and early postimplantation mouse embryos (JACKSON *et al.* 1981; FRANKE *et al.* 1982). The presence of certain cytokeratin polypeptides, especially the basic components higher than 60 kD suggested to be involved in keratinization (TSENG *et al.* 1982), may reflect the common histogenetic origin of the pseudostratified epithelium and the epidermis. Such a conclusion has already been drawn from studies showing the presence of antigenic determinants common to keratin polypeptides and loop arrays in certain neurons of the adult murine central nervous system (MASTERS *et al.* 1985). Ependyma cells were also stained by those anti-keratin antibodies which reacted with the loop arrays. The existence of a monoclonal antibody defined antigenic determinant common only to 57 kD and 65-67 kD epidermal keratins and the 26.5 kD keratinization associated protein, filaggrin (DALE & SUN 1983), supports the suggestion that the same polypeptides may be expressed during keratinization and differentiation of the neuroectoderm.

ZUSAMMENFASSUNG

Das Zytoskelett der Zellinie 9.5 BC, welche aus dem embryonalen Gehirn der Maus stammt, und isolierter Mesenzephalon-Rhombenzephalon-Proben von 10½ Tage alten Embryonen der Maus wurde mit Hilfe von Immunhistochemie, elektrophoretischer Trennung und Immunblotting untersucht. Die Zellinie besteht zu einem hohen Prozentsatz aus Zellen, welche positiv sind bezüglich des sauren fibrillären Glia-Proteins, und in geringerem Ausmass aus Zellen, welche positiv sind hinsichtlich des basischen Myelin-Proteins. Aus diesem Grunde scheint es sich um primitive Gliazellen zu handeln. Das Zytoskelett der proliferierenden Zellen besteht aus Aktin, Vimentin und mehreren Zytokeratinen (sauren Polypeptiden von 54 und 57 kD und einem basischen von 61 kD). Das Zytoskelett des embryonalen Gehirns besteht aus mehreren Zytokeratinen (sauren Polypeptiden von 40 und 46 kD, sowie basischen von 54, 57, 61, 63 und 68 kD), von denen das saure Zytokeratin von 46 kD mengenmässig vorherrscht. Vimentin ist bereits zwischen dem 10. und 11. Tag der Embryonalentwicklung nachweisbar. Da die Gliazellen des Zentralnervensystems aus einer Zytokeratin-positiven Zellpopulation entstehen und die Ausstattung ihres Zytoskeletts dann auf einen Zytokeratin-negativen Phänotyp umprogrammieren, sollte eine umfassende Erklärung für diesen histogenetischen Vorgang unter anderem auch die Repression der Polypeptid-Synthese der Zytokeratine im Verlaufe der Ontogenie berücksichtigen. Da es sich bei der Zellinie 9.5 BC um einen Zelltyp aus embryonalem Gehirn handelt, der über die potentielle Differenzierungsmöglichkeit verfügt Gliazellen hervorzubringen, kann man diese Zellinie als *in vitro*-Modell dieser Entwicklungsstufe betrachten.

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